Same article in Library set.

CHEMICAL STUDIES ON BLOOD.

I. ON THE COLORIMETRIC ESTIMATION OF GUANIDINE BASES IN BLOOD.

II. ON THE ACID HYDROLYSIS OF PROTEIN-FREE BLOOD AND TISSUE FILTRATES.

BY

JOSEPH JOHN PFIFFNER.

B.S., State University of Iowa, 1925.

M.S., State University of Iowa, 1926.

DISSERTATION

SUBMITTED TO THE GRADUATE COLLEGE OF THE STATE UNIVERSITY OF IOWA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

DEPARTMENT OF CHEMISTRY

JULY, 1928

3781
I65P

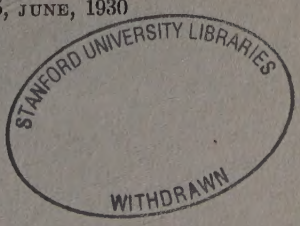
REPRINTED FROM

THE JOURNAL OF BIOLOGICAL CHEMISTRY

VOL. LXXXIII, NO. 2, PP. 289–297, AUGUST, 1929;

VOL. LXXXVII, NO. 2, PP. 345–355, JUNE, 1930

UNCLASSED



CHEMICAL STUDIES ON BLOOD.

I. ON THE COLORIMETRIC ESTIMATION OF GUANIDINE BASES IN BLOOD.

II. ON THE ACID HYDROLYSIS OF PROTEIN-FREE BLOOD AND TISSUE FILTRATES.

BY

JOSEPH JOHN PFIFFNER.

B.S., State University of Iowa, 1925.

M.S., State University of Iowa, 1926.

DISSERTATION

SUBMITTED TO THE GRADUATE COLLEGE OF THE STATE UNIVERSITY OF IOWA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

DEPARTMENT OF CHEMISTRY

JULY, 1928

REPRINTED FROM

THE JOURNAL OF BIOLOGICAL CHEMISTRY

VOL. LXXXIII, NO. 2, PP. 289–297, AUGUST, 1929;

VOL. LXXXVII, NO. 2, PP. 345–355, JUNE, 1930

Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. LXXXVII, No. 2, June, 1930

ON THE COLORIMETRIC ESTIMATION OF GUANIDINE BASES IN BLOOD.*

BY J. J. PFIFFNER AND VICTOR C. MYERS.

(From the Department of Biochemistry, State University of Iowa, Iowa City.)

(Received for publication, March 8, 1930.)

In 1924 Tiegs reported a new color reaction for the guanidine bases. On the basis of this reaction Marston (1924) elaborated a reagent for the colorimetric estimation of guanidine bases in pure solution. This reagent consisted of an alkaline peroxide-ferrocyanide-nitroprusside mixture which is by no means specific for the guanidine bases. Uric acid, ammonia, and most reducing substances inhibit the color reaction. Creatine and creatinine produce a color with the reagent. A short time later Marston (1925) pointed out that in estimating the guanidine bases in tissues ammonia may be removed by permutit and uric acid by precipitation with silver lactate but no details of the method were given. Whitehorn (1923) had already shown, however, that permutit removes guanidine from an aqueous solution of guanidine hydrochloride, and Pfiffner and Myers (1926) observed that permutit also removes methylguanidine and *as*-dimethylguanidine from aqueous solution, although the removal is not quantitative. A study of the reagent was undertaken in this laboratory with the object of applying it to the colorimetric estimation of guanidine bases in blood. In a preliminary communication Pfiffner and Myers (1926) described a modified peroxide-ferrocyanide reagent which kept well and gave within 5 minutes a full color development and did not fade or become turbid for more than an hour. Weber (1927) described a reagent for the colorimetric estimation of guanidine bases in which he substituted potassium ferricyanide for the potassium ferrocyanide and hydrogen peroxide of Marston's reagent. This substitution doubled the sensitivity of the reagent to guanidine bases. Major and Weber (1927) applied the reagent to the colorimetric estimation of guanidine bases in blood, and have made several reports of their various observations dealing particularly with hypertension.

In connection with observations on the retention of creatinine in nephri-

---

* The data here presented are taken from a dissertation submitted by Joseph J. Pfiffner to the Graduate College of the State University of Iowa, June, 1928, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

tis (Myers, 1914; Myers and Fine, 1914), it was pointed out that it was quite possible that this might be followed by transformation to the toxic methylguanidine. The following year Foster (1915) announced the isolation of a toxic substance from the blood of uremic patients as a gold salt although he made no statement as to its nature. At about this time some work was done by one of us (M.) in an effort to isolate methylguanidine from the blood of nephritic patients, but owing to apparently justified criticisms of the method employed (Baumann and Ingvaldsen, 1918; Greenwald, 1919) the work was abandoned.

With the appearance of the color reaction of Marston (1925) the work was resumed. Our findings (Pfiffner and Myers, 1926) indicated that the methylguanidine content of normal blood did not exceed 0.2 mg. per 100 cc., but the figures obtained on some pathological bloods were comparatively high. Although we had considered the question of the interference produced by creatinine, creatine, and urea, determined their color value and the quantities present in the various specimens, we ultimately concluded that we had underestimated the importance of these contaminants in the color reaction and decided to discard our results. Shortly after the publication of our preliminary report, Weber (1927) reported a study of the Marston color reaction, which has been followed by several papers by Major and Weber (1927) and Major (1929), in which they have presented considerable data showing that in patients with nephritis, and with arterial hypertension without nitrogen retention, there is a definite increase in the color reaction ascribed to the guanidines, the highest figures being found in the nephritic group.

In a very interesting and suggestive paper, Minot and Cutler (1928) have recently called attention to the presence of guanidine in the blood of dogs suffering from carbon tetrachloride and chloroform poisoning and to the antagonistic and beneficial action of calcium in this condition and in guanidine poisoning. With the development of the toxemia, a fall in the blood sugar and a rise in the icteric index was observed. In a subsequent preliminary report, Minot and Cutler (1929) have noted somewhat similar findings in acute liver injury and eclampsia.

Without entering into a further discussion of the subject, suffice it to say that there are a number of observations, in which a low blood calcium, and in some instances a low blood sugar, has been found to be associated with the presence of "guanidine compounds" in the blood, indicating the importance of this determination.

The object of the present paper is to record certain observations relative to the colorimetric reaction and to describe a modification of Weber's method which makes the use of a correction factor for blood creatine unnecessary.

The peroxide-ferrocyanide and the alkaline ferricyanide reagents have certain advantages and disadvantages. Both reagents pro-

duce an orange-red color with guanidine base[1] and a red color with methylguanidine[1] and *as*-dimethylguanidine.[1] With the peroxide-ferrocyanide reagent *as*-dimethylguanidine produces about 85 per cent of the color produced by methylguanidine, whereas guanidine produces 66 per cent. With the alkaline ferricyanide reagent *as*-dimethylguanidine produces 92 per cent of the color produced by methylguanidine, whereas guanidine produces only 40 per cent. These figures do not quite agree with those of Weber (1927) who found that guanidine and methylguanidine gave the same color value but dimethylguanidine only two-thirds as much. It appears fortunate that methylguanidine and *as*-dimethylguanidine have practically the same color value since it is expected that these two derivatives would be found in biological material rather than guanidine itself.

The alkaline ferricyanide reagent has the disadvantage of being very sensitive to the influence of salts. 20 mg. of sodium chloride depress the color development of 0.2 mg. of methylguanidine 20 per cent, whereas this amount of sodium chloride has no effect when the color is developed with the peroxide-ferrocyanide reagent. Ammonia and methylamine depress color development with both reagents. Color depression with ammonia is about 4 or 5 times greater with the peroxide-ferrocyanide reagent than with the alkaline ferricyanide reagent. The presence of excess alkali inhibits color development with either reagent. With the peroxide-ferrocyanide reagent the methylguanidine equivalent of 1 mg. of urea is 0.011; 1 mg. of creatinine, 0.005; 1 mg. of creatine, 0.22. With the alkaline ferricyanide reagent the methylguanidine equivalent of 1 mg. of urea is 0.006; 1 mg. of creatinine, 0.013; 1 mg. of creatine, 0.22. Time was allowed for full color development. Creatine develops a full color in about 5 minutes whereas urea and creatinine require about 15 minutes. The same color equivalents are displayed in the presence of a guanidine base.

[1] Standard solutions of guanidine hydrochloride, methylguanidine sulfate, and *as*-dimethylguanidine sulfate containing 1 mg. of the respective base per cc. were prepared by dissolving 160.2 mg. of guanidine hydrochloride, 167.1 mg. of methylguanidine sulfate, or 156.3 mg. of *as*-dimethylguanidine sulfate in distilled water and diluting to 100 cc. Diluted standards of the above three compounds were made by dilution with distilled water so that 10 cc. of solution contained 1 mg. of the respective base. Fresh standards were made up each week.

Both reagents react with arginine, histidine, and cysteine. Cystine gives no reaction. Arginine gives a rose-red color while histidine and cysteine give a green color. Histamine gives a green to reddish brown color depending on the amount present. Adrenalin and some of the alkaloids such as physostigmine, pilocarpine, and pelletierine give a red color. Procaine gives a green color. On the other hand methyl urea, β-methyl hydantoin, β-methyl hydantoic acid, sodium glycocholate, and thioneine do not give a color reaction.

The alkaline oxidation product of sodium nitroprusside responsible for color development with guanidine bases in the presence of ferricyanide can be obtained in dry stable form by precipitation from the oxidation mixture with ethyl alcohol. The method of obtaining the substance is as follows: Dissolve 5 gm. of sodium nitroprusside in 100 cc. of distilled water. Add 25 cc. of 40 per cent sodium hydroxide and while cooling in an ice bath add 30 per cent H_2O_2 slowly with constant stirring until a maximum green color is attained. Then add slowly 10 cc. more of 30 per cent H_2O_2. Transfer to a separatory funnel and add with shaking 700 cc. of 95 per cent ethyl alcohol. A heavy brown oil separates to the bottom. Draw off the precipitate and after dissolving in about 100 cc. of distilled water reprecipitate with the addition of 7 volumes of 95 per cent ethyl alcohol. After separating, dry the precipitate *in vacuo* over phosphorus pentoxide. A yellow amorphous water-soluble powder is obtained. The yield is about 5 gm.

In several different lots the nitrogen content varied from 9.5 to 11.5 per cent. If a few drops of a 1 per cent solution are added to a solution of a guanidine base (guanidine, methylguanidine, or *as*-dimethylguanidine) containing 0.05 mg. per cc., followed by a drop of 10 per cent potassium ferricyanide solution a deep red color develops immediately. No color is developed if ferrocyanide is used instead of ferricyanide. If the ferricyanide is added to the solution of the guanidine base *before* the oxidized nitroprusside product the color develops very slowly and requires about 1 hour for full color development compared to about 2 minutes when the reagents are added in the reverse order. If the 10 per cent potassium ferricyanide solution and the 1 per cent solution of oxidized nitroprusside product are first mixed in equal parts and then

added to the solution of a guanidine base there is no color development for 30 minutes and then a faint red color begins to develop. Sodium cyanide discharges the color of the reagents as well as the color formed by the interaction of the reagents with guanidine bases.

It would appear that the color formation is due to a reaction between the guanidine base, an alkaline oxidation product of sodium nitroprusside, and potassium ferricyanide in the order named. In the peroxide-ferrocyanide reagent the ferricyanide radical is apparently furnished by the oxidation of ferrocyanide, the alkaline peroxide oxidizing the sodium nitroprusside. In the alkaline ferricyanide reagent the nitroprusside is oxidized with alkaline ferricyanide and the ferricyanide radical having been added in excess is present in sufficient concentration for the color reaction.

In confirmation of Weber (1927) it was found that norit adsorbs guanidine bases quantitatively from a slightly alkaline solution and liberates them almost completely when treated with acidified alcohol. In a series of recoveries of guanidine bases from pure aqueous solution by adsorption on blood charcoal (Merck's purified by acid) under the conditions described by him we were able to recover methylguanidine on an average to the extent of 88.7 per cent (extreme variations 79 to 95 per cent), guanidine, 77.5 per cent (extreme variations 75 to 80 per cent), and *as*-dimethylguanidine 82.5 per cent (extreme variations 75 to 90 per cent). The adsorption is complete, low recoveries being accounted for because of incomplete release by acid alcoholic treatment. Norit does not adsorb guanidine base from neutral aqueous or absolute alcoholic solution although it does adsorb variable amounts of methylguanidine and *as*-dimethylguanidine under those conditions. Charcoal has the great disadvantage of adsorbing and releasing creatine under the same conditions as guanidine bases. Kaolin does not adsorb creatine and from this standpoint would be the ideal adsorbent, but it fails in that it does not adsorb the guanidine bases quantitatively. Adsorption of methylguanidine with Lloyd's reagent was only 75 per cent complete.

Since creatine is adsorbed by blood charcoal and released with acid alcoholic treatment it would interfere in the determination of guanidine bases in the blood and consequently Weber (1927)

introduced a correction factor for the amount of creatine present. This necessitated a determination of creatine and creatinine in the blood extract just prior to developing the color for the blood guanidine determination. The correction could not be based on the creatine content of the blood because, during the course of the determination, an acid solution of the blood extractives is concentrated on the water bath, converting some of the creatine to creatinine.

Since the obvious use of the method under discussion is to demonstrate the presence or absence of guanidine-like substances in the blood in certain pathological conditions it seems desirable to estimate the guanidine bases directly rather than by difference through the use of a correction factor for creatine. The use of a correction factor appears questionable due to the variation in percentage recovery of guanidine bases both from pure solution and from blood.

The logical solution is the conversion of creatine to creatinine by acid hydrolysis. It would be more convenient to autoclave the Folin-Wu filtrate before treatment with charcoal but the urea present would interfere. Urea is not adsorbed by charcoal in alkaline solution but autoclaving in acid solution would hydrolyze the urea and the ammonia formed would be adsorbed and subsequently released. Small amounts of ammonia interfere with color development. Hence in the described modification of Weber's method creatine is converted to creatinine after it is released from the charcoal. The percentage recovery with the modified direct technique is about the same (average 75.5 per cent) as the percentage recovery (average 76.2 per cent) obtained in a series of recoveries with Weber's (1927) method corrected for creatine. In our hands the variation in percentage recovery was the same in either case. It appears that some substance or substances are present in the final residue which tend to inhibit color development since guanidine bases added to the blood residue immediately before color development do not give their full color reaction. The chloride content of the residue is doubtless one factor since the average blood residue contains about 15 mg. of chloride calculated as sodium chloride. With the alkaline ferricyanide reagent 20 mg. of sodium chloride depress the color development of 0.2 mg. of methylguanidine as much as 20 per cent.

Method.

The modified method is as follows: 50 cc. of Folin-Wu blood filtrate are pipetted into a 150 cc. Erlenmeyer flask. 3 to 4 drops of 10 per cent NaOH are added followed by 0.5 gm. of blood charcoal (Merck's purified by acid). The contents of the flasks are mixed and filtered through a moist filter (Whatman No. 40). The flask is thoroughly drained. The filter is allowed to drain for

TABLE I.

Recovery of Methylguanidine from Blood.

Blood sample No. (1)	Methyl-guanidine added to 10 cc. of blood.* (2)	Methyl-guanidine as determined. (3)	Percentage recovery, uncorrected. (4)	Methyl-guanidine recovered, corrected.† (5)	Percentage recovery, corrected. (6)
	mg.	*mg.*		*mg.*	
1	0.40	0.34	85	0.32	80
	0.40	0.32	80	0.30	75
	0.20	0.16	80	0.14	70
2	0.10	0.10	100	0.08	80
	0.05	0.06	120	0.04	80
3	0.10	0.09	90	0.07	70
	0.08	0.075	94	0.055	69
	0.05	0.06	120	0.04	80
4	0.04	0.05	125	0.03	75
	0.06	0.06	100	0.04	67
	0.08	0.085	106	0.065	81
	0.10	0.11	110	0.09	90
Average			100.8		75.5

* Mixed human non-hypertensive pathological blood. Normal blood chemistry.

† Correction of 0.2 mg. of methylguanidine per 100 cc. of blood.

5 minutes and then returned to the original flask. 25 cc. of 95 per cent ethyl alcohol containing 0.5 cc. of N HCl are added to the contents of the flask. The flask is tightly stoppered, shaken, and allowed to stand overnight. The alcoholic solution is then filtered (Whatman No. 40). 20 cc. of the filtrate are transferred to an evaporating dish and brought to dryness on a water bath between

80–90°. The residue is taken up in 10 cc. of 0.2 N HCl, the dish covered with tin-foil (deep Pyrex dishes were used), and autoclaved for 30 minutes at 120°. The contents of the dish are again brought to dryness on a water bath between 80–90°. The last traces of HCl are dissipated by the addition of 1 or 2 cc. of absolute alcohol and subsequent evaporation. The residue is taken up in 4 cc. of distilled water and 1 cc. of alkaline ferricyanide reagent is added. Methylguanidine standards are made up simultaneously.

TABLE II.

"Guanidine" in Non-Hypertensive Blood Samples.

Subject.	Age.	Sex.	Blood chemistry.			Methyl-guanidine.	Diagnosis.
			Uric acid.	Urea N.	Creatinine.		
	yrs.		*mg. per 100 cc.*	*mg. per 100 cc.*	*mg per 100 cc.*	*mg. per 100 cc.*	
E. W. M.	24	M.				0.2	Normal.
J. J. P.	24	"				0.3	"
P. W. S.	42	"				0.2	"
D. B. M.	28	"				0.2	"
C. L.	68	"	6.9	14.2	1.1	0.2	Perforating ulcer of duodenum.
E. T.	31	"	3.5	16.8	1.1	0.3	Psychoneurosis, neurosthenia.
S. B.	56	"	3.9	11.8		0.3	Psychoneurosis.
L. G.	40	F.	2.4	10.6	2.5	0.2	Neurosis, menopause.
C. R.	14	M.	3.4	13.0	2.0	0.2	Hematuria.
M. R.	44	F.	3.8	10.4	1.7	0.2	Chronic cystitis.
Average.....						0.23	

The solutions are centrifuged and color comparison made within the second 5 minutes after addition of the reagent.

A series of methylguanidine standards containing 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, and 0.2 mg. of methylguanidine base is made up in matched test-tubes (12 × 100 mm., and marked at the 4 cc. level) from a solution containing 1 mg. of methyl guanidine base per 10 cc. and diluted with distilled water to the 4 cc. mark. To each is added 1 cc. of the alkaline ferricyanide reagent. If by inspection the unknown indicates a concentration of methylguanidine greater than 0.07 mg., it is matched against a

0.2 mg. standard in a Bausch and Lomb micro colorimeter, and the amount of methylguanidine present determined by reference to a proportionality curve. If the unknown indicates a concentration of less than 0.07 mg. the amount present is estimated by inspection. The amount found represents the amount in 4 cc. of

TABLE III.

"Guanidine" in Hypertensive Blood Samples.

Subject.	Age.	Sex.	Blood chemistry.			Blood pressure.	Methylguanidine.	Diagnosis, remarks.
			Uric acid.	Urea N.	Creatinine.			
	yrs.		*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mm. Hg*	*mg. per 100 cc.*	
J. G.	67	M.	5.7	21.7	1.4	150/80	0.2	Duodenal ulcer.
E. L.	16	F.	4.2	18.4	1.1	160/125	0.4	Mixed nephritis.
M. G.	16	"	4.0	12.0	1.0	148/74	0.2	Acute "
S. S.	54	"	4.4	12.8	1.1	135/90	0.3	Chronic colitis, chronic cholecystitis.
D. B.	62	M.	4.6	15.4	1.1	248/118	0.6	Chronic interstitial nephritis.
W. S.	62	"	4.7	21.0	1.6	190/130	0.3	Arteriosclerosis, cerebral double hemiplegia, dementia.
						190/130	0.4	Second determination, 6 days later.
A. S.	62	M.				150/80	0.4	Neurosyphilis, chronic progressive vascular nephritis; duplicate determinations.
							0.4	
W. P.	68	M.	3.3	14.7	3.6	158/76	0.2	Enlargement of prostate, hypertrophy, diverticula of bladder, chronic cystitis.
Average							0.33	

blood. Hence the amount found multiplied by 25 gives the amount of methylguanidine in 100 cc. of blood.

The results of a series of recoveries of methylguanidine added to human blood are recorded in Table I.

The color produced by the blood residue itself corresponds

to 0.2 mg. of methylguanidine per 100 cc. of blood. When the correction factor of 0.02 is applied per 10 cc. of blood (Column 5) the average per cent of recovery (Column 6) is 75.5 per cent.

Many control samples of blood were carried through the method side by side with samples having added methylguanidine. The color developed by quantities of methylguanidine less than 0.01 mg. is very slight. Due to this limitation in the method the best that can be said is that the control bloods contained less than 0.2 mg. of "guanidine" per 100 cc. of blood. The amount present may be actually 0.

A series of normal and hypertensive blood samples was analyzed with the technique described. The data are recorded in Tables II and III. There appears to be a slight but definite increase in the color reaction displayed by some hypertensive blood samples. It cannot be stated whether or not such increase is actually due to a guanidine base. These findings are in confirmation of those of Major and Weber (1927) and Major (1929) who found in cases of hypertension a content between 0.2 and 0.5 mg. of blood "guanidine" per 100 cc. of blood, while in cases of nephritis with considerable nitrogen retention the figures ranged from 0.3 to 2.3 mg.

SUMMARY.

1. A study has been made of the colorimetric reaction of certain guanidine bases with alkaline oxidized nitroprusside and ferricyanide.

2. Weber's method (1927) for the estimation of guanidine bases in blood has been modified to exclude interference by creatine.

3. The modified method has been applied to a few normal and hypertensive blood samples.

BIBLIOGRAPHY.

Baumann, L., and Ingvaldsen, T., *J. Biol. Chem.*, **35**, 277 (1918).

Foster, N. B., *Tr. Assn. Am. Phys.*, **30**, 305 (1915).

Greenwald, I., *J. Am. Chem. Soc.*, **41**, 1109 (1919).

Major, R. H., *Am. J. Med. Sc.*, **177**, 188 (1929).

Major, R. H., and Weber, C. J., *Bull. Johns Hopkins Hosp.*, **40**, 85 (1927); *Arch. Int. Med.*, **40**, 891 (1927).

Marston, H. R., *Australian J. Exp. Biol. and Med. Sc.*, **1**, 99 (1924); **2**, 57 (1925).

Minot, A. S., and Cutler, J. T., *J. Clin. Inv.*, **6**, 369 (1928); *Proc. Soc. Exp. Biol. and Med.*, **26**, 607 (1929).
Myers, V. C., *Post-Grad. Med. J.*, **29**, 513 (1914).
Myers, V. C., and Fine, M. S., *Proc. Soc. Exp. Biol. and Med.*, **11**, 132 (1914).
Pfiffner, J. J., and Myers, V. C., *Proc. Exp. Biol. and Med.*, **23**, 830 (1926).
Tiegs, O. W., *Australian J. Exp. Biol. and Med. Sc.*, **1**, 93 (1924).
Weber, C. J., *Proc. Soc. Exp. Biol. and Med.*, **24**, 712 (1927).
Whitehorn, J. C., *J. Biol. Chem.*, **56**, 751 (1923).

II. ON THE ACID HYDROLYSIS OF PROTEIN-FREE BLOOD AND TISSUE FILTRATES.*

*The data in abstract form are incorporated in the following paper with data taken from the dissertation submitted by R. G. Turner to the Graduate College of the State University of Iowa, 1927, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Reprinted from The Journal of Biological Chemistry
Vol. LXXXIII, No. 2, August, 1929

A PREVIOUSLY UNDETECTED CONSTITUENT OF BLOOD.

By E. W. ROCKWOOD, R. G. TURNER, and J. J. PFIFFNER.

(From the Laboratory of Biochemistry, University of Iowa, Iowa City.)

(Received for publication, April 23, 1929.)

In protein-free tungstic acid blood filtrates, besides uric acid, are other substances which give a blue color with alkaline arsenophosphotungstate. Bulmer, Eagles, and Hunter (1) demonstrated the presence of such a substance. It was subsequently isolated by Hunter and Eagles (2, 3) and was shown to be ergothioneine by Benedict and coworkers (4, 5, 6) and Eagles and Johnson (7). The name thioneine was suggested by Benedict (6) for the substance.

Acid hydrolysis of the protein-free filtrate liberates uric acid (Benedict and coworkers (8–11)). Evidence is here presented that acid treatment of the thioneine fraction increases the material measured by the arsenophosphotungstate reaction. Provisionally the substance so produced has been named substance Z.

Since uric acid, thioneine, and substance Z give the same blue color it seemed desirable to determine their quantities. Oxalated blood was used in all cases and four colorimetric determinations were made on each sample; (1) uric acid direct,[1] (2) uric acid indirect, (3) thioneine plus substance Z, (4) thioneine. A part of the blood filtrate was hydrolyzed with sulfuric acid, a part was not so treated; uric acid, both direct and indirect, was determined in the unhydrolyzed portion. Substance Z is freed by acid hydrolysis and is then precipitated by silver lactate but is not afterwards liberated by acid NaCl as used by Folin and Wu (12). Hence it is determined with thioneine in the silver residue from the hydrolyzed portion. Thioneine is similarly determined in the unhydrolyzed portion.

Other than the usual reagents for uric acid determination there

[1] The terms "direct" and "indirect" are used with the significance given them by Bulmer, Eagles, and Hunter (1).

are: (1) concentrated sulfuric acid diluted 1:10 by volume; (2) ammoniacal sodium acetate solution, containing 300 cc. of concentrated NH_4OH with 700 cc. of 4 N $NaC_2H_3O_2$; (3) silver lactate solution. Mix 50 cc. of 10 per cent NaOH and 50 cc. of 85 per cent lactic acid and add to 50 gm. of silver lactate (Merck) previously dissolved in 350 cc. of warm water. Bring to 1000 cc. and keep in the dark for at least 6 weeks before using. It must be clear and colorless.

Procedure.

To 20 cc. of Folin and Wu blood filtrate in a 50 cc. centrifuge tube add 7 cc. of silver lactate solution. Let it stand 2 or 3 minutes, centrifuge, and decant the supernatant liquid. To the residue add 1 cc. of 10 per cent NaCl in 0.1 N HCl, stir with a small glass rod, add 4 cc. of water, and centrifuge again. Decant the supernatant liquid into a clean, dry test-tube. Repeat the treatment with acid NaCl and water and the centrifugation, adding the liquid to the portion previously obtained. Dilute this mixture with distilled water to 20 cc., the volume of the original blood filtrate. Transfer 5 cc. of the mixed liquid to another clean, dry test-tube, add 5 cc. of water, 4 cc. of 5 per cent NaCN, 1 cc. of Benedict's uric acid reagent, and develop the color in the usual manner. This represents indirect uric acid. Direct uric acid is determined in the Folin and Wu blood filtrate according to Benedict (13).

For the thioneine determination add 4 cc. of 5 per cent NaCN to the silver residue from the second treatment with acid NaCl and stir until completely dissolved. Decant into a clean, dry test-tube; rinse twice with 5 cc. portions of water, adding each portion to the decanted cyanide, then add 1 cc. of uric acid reagent and carry out the colorimetric determination as for uric acid. A 0.02 mg. standard is generally used though sometimes a 0.01 mg. one (Benedict (13)) is preferable. The standard tube is set at 15 and the thioneine, in terms of uric acid, is calculated by $\frac{15}{R} \times 0.02 \times \frac{100}{2} = \frac{15}{R}$ = mg. of thioneine in 100 cc. of blood when R is the reading of the unknown and 0.02 mg. the uric acid standard used.

For the determination of thioneine plus substance Z 15 cc. of

Folin and Wu blood filtrate are transferred to a test-tube (1 × 8 inches) for hydrolysis and 3 cc. of the diluted sulfuric acid are added. As an air condenser a glass tube (200 cm. × 8 mm.) is attached to the test-tube by a 1-hole rubber stopper. The contents of the tube are gently boiled for 15 minutes over a low flame (a micro burner may be used). After cooling, giving time for complete drainage of the condenser before disconnecting, shake so as to wash down any liquid which may have condensed in the upper part of the test-tube. The volume of the liquid must be the same as before hydrolysis, 18 cc.

To the hydrolyzed blood filtrate add from a 10 cc. burette 2 to 3 cc. of the ammoniacal sodium acetate solution. The amount required is previously found by titrating 15 cc. of protein-free filtrate mixed with 3 cc. of the sulfuric acid until faintly alkaline to Congo red. 2.5 cc. of the acetate solution will usually be found correct; it is best to check it occasionally especially after preparing new reagents for precipitation of the blood proteins. Mix and pour into a centrifuge tube, rinsing with 5 cc. of water. Treat this solution as described in the method for the determination of indirect uric acid and thioneine except that the acid NaCl washings are discarded and thioneine plus substance Z determined from the silver residue remaining. Amounts are calculated from $\frac{15}{R} \times 0.02 \times \frac{100}{1.5}$ = mg. of thioneine + substance Z in 100 cc. of blood where R is the reading of the unknown and the 0.02 mg. standard is set at 15 mm. Subtracting the amount of thioneine found in the unhydrolyzed filtrate from the above gives the amount of substance Z.

The development of color in all four determinations is made at the same time and compared against the same standard. If read immediately after cooling no turbidity results. The maximum limit of error permitted in duplicate determinations was 0.1 mg. in 100 cc.

The blood was drawn from the cubital vein, oxalated, and treated immediately with tungstic acid. Ample time was allowed for complete precipitation. The results for fifteen normal bloods are given in Table I. They are expressed (as are all others cited) in terms of mg. of uric acid in 100 cc. of blood.

Of the thirty-eight normal human bloods examined only one

TABLE I.

*Uric Acid, Thioneine, and Substance Z in Normal Human Blood.**

Subject No.	Uric acid.		Thioneine.	Substance Z.
	Direct method.	Indirect method.		
1	4.4	3.2	1.1	0.5
2	3.0	2.4	0.6	0.7
3	3.6	2.6	1.1	0.4
4	4.3	3.3	0.8	0.5
5	3.5	2.9	0.7	1.1
6	3.6	3.5	0.0	0.9
7	4.0	4.0	0.0	0.5
8	3.3	2.8	0.7	0.3
9	3.5	3.3	0.7	0.2
10	3.4	3.2	0.8	0.3
11	4.2	3.8	1.1	0.6
12	3.2	3.2	0.5	0.4
13	2.7	2.3	0.9	0.4
14	2.9	2.6	0.7	0.0
15	3.6	3.5	0.8	0.4
Average.....	3.5	3.1	0.7	0.5
" of 38 samples.....	3.3	2.8	0.7	0.5

* Results are expressed as mg. of uric acid per 100 cc. of blood.

TABLE II.

Thioneine and Substance Z in Pathological Human Blood.

Diagnosis.	No. of cases.	Thioneine.		Substance Z.	
		Range.	Average.	Range.	Average.
Pernicious anemia.....	8	0.4–1.0	0.6	0.2–1.8	1.2
Leucemia.....	3	0.7–0.8	0.7	0.7–1.8	1.1
Nephritis.....	4	0.5–1.0	0.7	0.0–1.0	0.4
Diabetes mellitus.....	4	0.8–1.2	1.0	0.6–1.3	0.9
Arsenical dermatitis.....	3	0.8–1.2	1.0	0.1–1.6	0.8
Primary lues.....	9	0.5–1.0	0.7	0.1–1.4	0.6
Secondary "	9	0.6–1.0	0.7	0.1–1.0	0.4
Tertiary "	4	0.5–0.9	0.6	0.1–1.5	0.8
Latent "	4	0.7–1.0	0.8	0.2–1.5	0.7
Paresis.....	2	0.5–0.4	0.4	0.8–0.8	0.8

failed to give a test for substance Z. Twenty-nine contained more than 0.2 mg. (in terms of uric acid) in 100 cc. Eighteen showed 0.5 mg. or more; the highest was 1.1 mg. It will be seen that the level of substance Z is not related to the thioneine or uric acid content, nor is it related to age or sex, though in most

TABLE III.

Uric Acid, Thioneine, and Substance Z in Animal Blood and Tissues.*

Species.	Tissue.	Uric acid.	Thioneine.	Substance Z.	Species.	Tissue.	Uric acid.	Thioneine.	Substance Z.
Dog I	Blood.	1.0	0.4	0.6	Rabbit XI	Muscle.	1.4	0.5	0.2
" II	"	1.2	0.5	0.2		Kidney.	2.9	1.1	0.2
" III	"	1.1	0.4	0.5		Liver.	8.0	1.0	0.8
" IV	"	1.1	0.4	0.1					
" V	"	1.1	0.5	0.6	" XII	Blood.	1.0	0.5	0.1
						Muscle.	1.3	0.4	0.3
" VI	Muscle.	2.7	1.3	0.5		Kidney.	2.8	1.5	0.3
	Liver.	6.0	2.1	0.5		Liver.	8.2	1.5	0.4
	Kidney.	6.7	4.7	0.5					
					" XIII	Blood.	1.2	0.8	0.2
"VII	Muscle.	3.7	2.4	0.2		Muscle.	1.6	0.5	0.2
	Liver.	6.4	3.5	1.2		Kidney.	2.9	1.2	0.1
	Kidney.	8.0	6.8	0.2		Liver.	5.4	1.3	0.5
Rabbit I	Blood.	1.3	0.6	0.2	Pig I	Blood.	1.0	0.6	0.1
" II	"	1.1	0.4	0.1	" II	"	1.5	0.9	0.2
" III	"	1.7	1.0	0.5		Liver.		1.9	0.6
" IV	"	1.4	0.6	0.1					
" V	"	1.5	0.5	0.8	Ox I	Blood.	2.0	0.5	0.2
					" II	"	2.1	0.5	0.1
					" III	"	1.8	0.4	0.2
					" IV	"	2.3	0.4	0.3

* As determined by Benedict's direct method.

cases the content of female blood is low. The distribution of substance Z between plasma and corpuscles appears to be equal.

The results of analyses of pathological bloods are shown in Table II.

The range of thioneine and substance Z content is recorded because of the belief that the average is of less significance. No path-

ological case was examined which gave consistently a high or low value for substance Z although in both pernicious anemia and leucemia the general trend was toward a high level. There was no correlation of these findings with either the white or the red blood cell counts. It is of interest to know that in nephritis the level of substance Z tends to be low, while not paralleling the non-protein nitrogen. In several cases of infectious lues the amount of substance Z in the blood decreased markedly with the administration of organic arsenicals, where it was high before treatment. Often where the amount of substance Z was 0.6 mg. per 100 cc. or less no such change occurred. In diabetes the substance Z content of the blood did not parallel the blood sugar level. In this connection it may be noted that addition of urea and dextrose to the blood in five or six times the normal amount does not influence the substance Z content. Rabbits infected with *Treponema pallidum* showed a substance Z content of the blood no different from the normal determined before infection. The arsenicals, arsphenamine and stovarsol, when administered to rabbits failed to produce any change in substance Z content.

For comparison with human blood similar determinations were made on animal bloods; their tissues were also tested. Dog and rabbit blood was obtained by cardiac puncture; pig and ox blood came fresh from the abattoir as did also pig liver. All tissue filtrates were prepared from the still warm organs by the method of Folin, Berglund, and Derick (14). The results are recorded in Table III.

It is apparent that the amount of substance Z in either blood or tissue filtrates is in no way related to that of thioneine or uric acid (by the direct method). There is no distinct difference in its level in the blood of man, dog, or rabbit. In the few specimens of pig and ox blood examined the amount found was small. Muscle and kidney contain about equal amounts, liver considerably more. Severe liver injury produced with hydrazine sulfate in rabbits failed to alter the level of substance Z in any of the tissues.

The substance Z content of human blood is not altered by sterile or non-sterile incubation for 24 hours at 40°. As seen from the observations recorded in Table IV it increases during incubation of rabbit or pig liver without a preservative though no such

increase appeared in rabbit blood, muscle, or kidney. In incubation with a preservative a 5 gm. portion was ground with quartz sand, then suspended in water to 90 cc. under toluene.

DISCUSSION.

Substances which will reduce phosphotungstic acid reagent are not limited to uric acid and thioneine. Among such active sub-

TABLE IV.

Thioneine and Substance Z Fractions in Preserved and Incubated Liver Tissue.

Materials and conditions.	Thioneine fraction.	Substance Z.
Pig liver, fresh from abattoir	2.0	0.5
" " kept at 10° for 72 hrs	2.0	0.5
" " " " −5° " 72 "	2.4	0.5
" " incubated under toluene for 24 hrs. at 37°	3.6	0.6
" " " " " " 72 " " 37°	4.0	0.7
Pig liver, fresh from abattoir	1.9	0.6
Pig liver, incubated 24 hrs. at 37° without preservative	3.1	1.7
Pig liver, incubated 72 hrs. at 37° without preservative	2.7	1.4
Pig liver, kept at −5° for 72 hrs. then incubated 24 hrs. at 37° without preservative	2.7	1.4
Rabbit liver, fresh from animal	1.5	0.4
" " kept 72 hrs. at 10°	2.6	0.7
" " incubated 12 hrs. at 37°, no preservative	2.2	0.9
" " kept at 10° for 72 hrs. then incubated 12 hrs. at 37°	3.6	1.0

stances have been pointed out metallic silver (13), cystine after reduction to cysteine (15), and glutathione (16) as well as insulin (17).

It has been shown (12) that acid NaCl quantitatively removes uric acid from the silver precipitate; inasmuch as substance Z is not thus removed it cannot be uric acid freed by acid hydrolysis from some combined form. Since glutathione is present in the blood as such it is evident that substance Z is not glutathione.

The possibility must be considered that it is cystine or cysteine, liberated by acid hydrolysis from glutathione, a dipeptide of cysteine and glutaminic acid. Hunter and Eagles (18) found for such hydrolysis of glutathione that it was necessary to boil for 8 hours with 6 N sulfuric acid; for the development of substance Z the heating was for 15 minutes with 0.6 N acid. Hunter and Eagles (19) found normal human blood corpuscles to contain in 100 cc. about 100 mg. of glutathione; Thompson and Voegtlin (20) showed that it is absent from the serum. Whole blood therefore contains approximately 50 mg. of glutathione per 100 cc. This 50 mg. represents potentially 24.1 mg. of cystine. The results obtained with the Folin and Wu filtrate to which cystine

TABLE V.

Effect of Added Cystine and Cysteine on Thioneine Fraction of Blood.

Figures are in mg. per 100 cc. of blood.

Blood No.	Cysteine added.	Cystine added.	Thioneine.
1 a	0.0		0.6
1 b	25.0		0.8
1 c		25.0	0.8
2 a	0.0		0.7
2 b	25.0		0.9
2 c	37.5		1.0
2 d	50.0		1.1

or cysteine had been added show an increase of only 0.2 mg. (expressed in terms of uric acid) for 25 mg. added. Even if complete hydrolysis in 15 minutes were possible twenty-nine of the thirty-eight bloods examined contained more than 0.2 mg. of substance Z. Our distributional analysis showed no more substance Z in protein-free corpuscle filtrate than in plasma filtrate while glutathione does not occur in the latter (20). Sullivan's naphthoquinone test (21) was applied to the bloods of Table V. In the Folin and Wu filtrate, before or after hydrolysis it was negative; where cysteine was added it was positive. Consequently substance Z is neither glutathione nor cysteine split from it.

Du Vigneaud (17) obtained a blue color with hydrolysates of insulin materials but Shonle and Waldo (22) found that tungstic

acid filtrates contained no insulin, which eliminates that from consideration.

SUMMARY.

A previously undetected substance, reducing arsenophosphotungstic acid, is shown to be present after acid hydrolysis of the blood of man, dog, and rabbit. It is also found in the muscle, kidney, and liver of dog and rabbit. In the few ox and pig bloods examined it was detected only in traces.

It is provisionally named substance Z.

A method is described for its estimation in tungstic acid blood and tissue filtrates. Its amounts have been determined in normal and various pathological bloods.

It is not the same as any of the known constituents of tungstic acid blood and tissue filtrates.

BIBLIOGRAPHY.

1. Bulmer, F. M. R., Eagles, B. A., and Hunter, G., *J. Biol. Chem.*, **63**, 17 (1925).
2. Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, **65**, 623 (1925).
3. Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, **72**, 123 (1927).
4. Benedict, S. R., Newton, E. B., and Behre, J. A., *J. Biol. Chem.*, **67**, 267 (1926).
5. Newton, E. B., Benedict, S. R., and Dakin, H. D., *Science*, **64**, 602 (1926).
6. Newton, E. B., Benedict, S. R., and Dakin, H. D., *J. Biol. Chem.*, **72**, 367 (1927).
7. Eagles, B. A., and Johnson, T. B., *J. Am. Chem. Soc.*, **49**, 575 (1927).
8. Benedict, S. R., *J. Biol. Chem.*, **20**, 633 (1915).
9. Davis, A. R., Newton, E. B., and Benedict, S. R., *J. Biol. Chem.*, **54**, 595 (1922).
10. Newton, E. B., and Davis, A. R., *J. Biol. Chem.*, **54**, 601 (1922).
11. Newton, E. B., and Davis, A. R., *J. Biol. Chem.*, **54**, 603 (1922).
12. Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 81 (1919).
13. Benedict, S. R., *J. Biol. Chem.*, **51**, 187 (1922).
14. Folin, O., Berglund, H., and Derick, C., *J. Biol. Chem.*, **60**, 361 (1924).
15. Folin, O., and Looney, J. M., *J. Biol. Chem.*, **51**, 421 (1922).
16. Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, **72**, 177 (1927).
17. du Vigneaud, V., *Proc. Soc. Exp. Biol. and Med.*, **24**, 547 (1927).
18. Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, **72**, 147 (1927).
19. Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, **72**, 133 (1927).
20. Thompson, J. W., and Voegtlin, C., *J. Biol. Chem.*, **70**, 793 (1926).
21. Sullivan, M. X., *Pub. Health Rep., U. S. P. H. S.*, **41**, 1030 (1926).
22. Shonle, H. A., and Waldo, J. H., *J. Biol. Chem.*, **58**, 731 (1923–24).

BIOGRAPHY.

JOSEPH JOHN PFIFFNER was born at Dubuque, Iowa, on June 24, 1903. He received his grade school education in the parochial schools and his academic training in Columbia Academy of that city. In the fall of 1921, he enrolled in the College of Pharmacy of the State University of Iowa. The degrees of Graduate in Pharmacy, Pharmaceutical Chemist, and Bachelor of Science in Pharmacy were granted to him, the first in 1923, the remaining two in 1925. In the summer of 1925, he enrolled in the Graduate College of the State University of Iowa and was granted the degree of Master of Science in July of the following year. He served as a graduate assistant in chemistry during the school years 1926–27 nd 1927–28.

WAVERLY PRESS, INC.
BALTIMORE, U. S. A.